# GRAFTING OF VINYL MONOMERS ON TO MODIFIED COLLAGEN BY CERIC ION—STUDIES ON GRAFTING SITE

K. PANDURANGA RAO, K. THOMAS JOSEPH & Y. NAYUDAMMA
*Central Leather Research Institute, Madras*

Received on November 11, 1969

Graft polymerisation of methylmethacrylate on a number of modified collagens was studied in order to elucidate the mechanism of the oxidation of collagen and to determine the grafting sites on collagen using the ceric ion technique. The number of grafting sites obtained in the case of unmodified collagen indicated that grafting reactions involved only a small proportion of the fibre molecules. None of the modifications of the different functional groups of collagen could completely eliminate grafting, except dinitrophenylation; in this case the complete inhibition of grafting was due to the presence of aromatic nitro compounds. The number of grafting sites significantly increased when collagen was thiolated, N-brominated, vinylated, cyanoethylated or methylated. On the contrary, the number of grafting sites decreased when collagen was acetylated or treated with oxidised starch. The decrease in the number of grafting sites with oxidised starch treated collagen is attributed to the presence of free aldehyde groups in the modified collagen. The general trend of the results obtained with modified collagen samples indicate that hydroxy groups, amino groups and the peptide backbone may provide sites for initiation of grafting reaction.

Several detailed studies have appeared recently on the mechanism and kinetics of graft polymerisation of vinyl monomers on to polyhydroxyl substrates such as cellulose, starch, polyvinyl alcohol etc. using ceric ion initiation. However, very little information is available on the grafting of vinyl monomers on to proteins and the number of potential catalysts for graft copolymerisation treatment of proteins is limited. In proteins, where the sites are numerous and less accessible, large molecules will not diffuse into the protein fibres, particularly if they are soluble only in non-ionic solvents. Previous publications,[1,2] from this laboratory have presented evidence for the formation of graft copolymers when vinyl monomers are polymerised in an aqueous slurry of hide collagen by initiation with ceric ion. But the detailed mechanism of the oxidation of collagen whereby ceric salts initiate great copolymerisation is yet to be elucidated. Iwakura and Imai[3,4] have also reported the grafting of vinyl monomers on to proteins using ceric ion method.

In the present paper, an attempt was made to determine the grafting sites on

PMMA — Polymethylmethacrylate
CAN — Ceric Ammonium Nitrate

collagen. Collagen contains several functional groups such as hydroxyl, amino, carboxyl etc. which are capable of forming redox systems with ceric ion. In order to gain more insight into the nature of the amino acid residues involved in the attachment of the graft polymer, grafting experiments were carried out on a number of modified collagens.

## Experimental

(1) *Preparation and analysis of modified collagen*

Collagen was prepared from the butt portion of fresh buffalo hide. Deaminated,[5] methylated,[6] N-acetylated[7] and total acetylated[8] collagens were prepared and analysed as per the procedure reported earlier. Dinitrophenylation of collagen was carried out by the original method of Sanger.[9] After the reaction the protein was thoroughly washed with water and soxhlet extracted with ethanol for 12 hours. Cynoethylation of collagen was carried out by the method of Feairheller *et al.*[10]

*Thiolation of collagen*

In the present study, the method of Singer *et al*[11] was followed for the thiolation of collagen. 5 g. of collagen was suspended in 100 ml of carbonate buffer pH 10·7 and the suspension was cooled to 0°C. 1·25 g. of homocysteine thiolcatone dissolved in water was then added and the suspension was shaken for four hours, the temperature being maintained at 0°C. The collagen was then filtered, washed with distilled water and directly used for grafting studies. Analysis of the modified collagen showed that only about 50% of ε-amino groups were substituted.

*N-Bromination*

N-halogenated amides can be prepared from polyamides and polypeptides by direct halogenation. The method adopted by Bamford *et al*[12] for the N-bromination of polypeptides and proteins was followed. Collagen was N-Brominated by immersion for several minutes at room temperature in a solution of sodium hypobromite acidified to pH 4—4·5 with glacial acetic acid. It was then washed with distilled water and dried in vacuum at 40°C. Bamford *et al*[12] have shown that under these conditions gelatin takes up about 8·3% of bromine.

*Vinylation of collagen*

Vinyl groups were introduced in collagen by two different methods, namely by treatment with maleic anhydride[13,14] and also by acrolein.

Collagen (15 g) was suspended in 200 ml. of dimethyl formamide and 30 g. of maleic anhydride was added and the mixture was kept at room temperature for 3-4 days. The collagen was then filtered and deswelled with acetone. Treatment with acrolein was carried out by suspending collagen in carbonate buffer of pH 9 and treating with acrolein (3% solution) for 24 hours. After the reaction, the collagen was thoroughly washed and air dried.

*Treatment of collagen with dialdehyde starch*

Since it has been shown that ceric ion redox system is effective for the graft copolymerisation of vinyl monomers on polyhydroxy compounds such as cellu-

lose and starch, it was of interest to attach such polyhydroxy compounds on collagen and then study the graft polymerisation on the modified collagen. Collagen was therefore, treated with a 10% oxidised dialdehyde starch at pH 8-9 and the modified protein was washed and used for grafting studies.

*Grafting procedure*

All the grafting experiments were carried out in nitrogen atmosphere at the laboratory temperature as outlined in the previous papers.[1,2] The crude graft copolymer was extracted with dichloroethane for 72 hours and then with acetone for 24 hours at room temperature to remove the ungrafted homopolymer. The extracted dried product was then analysed for total nitrogen, arginine and hydroxyproline. Total nitrogen was determined by the Kjeldahl method, arginine by the method of Macpherson[15] and hydroxyproline by the method of Newman and Logan.[16] The collagen content of the graft copolymers was calculated from the arginine or nitrogen values.

The percentage weight increase was calculated as

$$\frac{\text{Total weight of graft copolymer} - \text{Weight of collagen}}{\text{Weight of collagen}} \times 100$$

The number of grafting sites per mole of collagen was calculated as

$$\frac{\text{Weight of PMMA/molecular weight of grafted PMMA/}}{\text{Weight of used collagen/molecular weight of collagen}}$$

*Hydrolysis of collagen—Methylmethacrylate graft copolymers*

Samples of collagen-methylmethacrylate graft copolymers (0·5—1·0 g.) were treated with 20 ml. of 6N hydrochloric acid at 100—105°C for 18 hours. The precipitates were filtered, washed with water and methanol, dried in vacuo and weighed.

*Molecular weight determinations*

The intrinsic viscosity of the isolated grafts was determined in benzene at 30°C using a PCL suspended Dilution Viscometer. The number of average molecular weight was calculated from the equation[17]

$$[\eta] = 8{\cdot}69 \times 10^{-5}\ \overline{M}_n^{0.76}$$

*Hexose residues of collagen as a possible site of grafting*

Collagen contains a small but definite amount of hexose residues and it is possible that these hexose residues may also act as site for the grafting of vinyl monomers on collagen. In the case of certain glycoproteins like Ovalbumin, it has already been reported[3] that the carbohydrate residues are involved in the grafting of vinyl monomers by the ceric ion method. To check this possibility in the case of collagen, grafting studies were made on soluble tropocollagen. A solution of tropocollagen in dilute acetic acid was grafted with methylmethacrylate under the usual conditions. A known weight of the graft copolymer was then hydrolvsed with 0·5N $H_2SO_4$ for 5 hours. The insoluble graft was removed by filtration and the filtrate

and washings were made upto a known volume and submitted to sugar analysis by the phenol-sulphuric acid method.[18] The amount of collagen in the graft copolymer was determined by nitrogen analysis. Ungrafted collagen was also analysed for sugar under identical conditions.

*Hydroxyproline in grafted collagen*

Since in the earlier studies[1,2] it was found that grafted collagen on acid hydrolysis gave lower values for hydroxyproline, it was necessary to find out whether the grafting medium has got any influence on the hydroxyproline content of collagen. For this, control experiments were carried out in which the collagen was treated with nitric acid of the same strength as used in the grafting studies, both alone and in the presence of ceric ion but without any vinyl monomer. Similarly, in order to ascertain whether the graft polymer has got any effect on hydroxyproline during hydrolysis, a known weight of collagen was mixed with an equal weight of PMMA and then hydrolysed with 6N HCl. The PMMA polymer was removed by filtration and the filtrate and washings were combined and analysed for hydroxyproline.

## Results and Discussion

From Table 1 it can be seen that in the case of collagen fibers, grafting reactions probably involve only a small proportion of the fibre molecules. Physical, chemical measurements and electron microscopy have shown that the collagen molecule can be represented approximately by a cylinder about 3000 Å long and 14 Å in diameter with a mole-

**Table 1**

GRAFTING OF METHYL METHACRYLATE ON TO MODIFIED COLLAGEN
(Reaction time: 3 hours at room temperature)

| Nature of modification | Percent grafting* | Mol. wt. of grafted PMMA × $10^{-5}$ | No. of grafting sites, mole/mole |
|---|---|---|---|
| Untreated collagen | 159·60 | 20·75 | 0·2301 |
| Acetylated „ | 93·28 | 16·94 | 0·1651 |
| Methylated „ | 159·40 | 11·48 | 0·4168 |
| N-Acetylated „ | 156·00 | 22·70 | 0·2071 |
| Deaminated „ | 51·07 | 7·55 | 0·2030 |
| Dinitrophenylated collagen | Nil | — | — |
| Thiolated „ | 146·00 | 2·95 | 1·485 |
| Cynoethylated „ | 183·90 | 14·16 | 0·3896 |
| N-Brominated „ | 108·40 | 6·29 | 0·5166 |
| Vinylated (Maleic anhydride) collagen | 301·90 | 18·32 | 0·4943 |
| Vinylated (Acrolein tanned) „ | 129·10 | 10·21 | 0·3793 |
| Dialdehyde starch tanned „ | 12·06 | 19·50 | 0·0185 |

*Calculated either from nitrogen analysis or arginine estimation.

cular weight of about 300,000. When forming fibrils, these collagen monomers arrange themselves in a staggered array so that an infinite structure in the direction of the length of the monomers is formed. The number of grafting sites obtained in the case of unmodified collagen indicates that one collagen molecule in four is only involved in the grafting reaction. The fact that the graft copolymers have only a small number of branches is fairly common in the case of graft copolymers prepared by a free radical mechanism. The molecular weight of the isolated grafts indicates that in most cases, the grafted chains are many times larger than the tropocollagen molecules making up the collagen fibres. Only in the case of thiolated collagen, the molecular weight of the isolated grafts was more or less of the same order as that of tropocollagen (300,000). In this case, the number of grafting sites was also the maximum. From the data available in the literature[19,20] carbon-halogen and sulphur-hydrogen bonds are more susceptible to radical attack and transfer than carbon-hydrogen bonds; and by incorporating halides or mercaptans into the backbone, the ratio of the chain transfer to chain propagation on which the efficiency of grafting process depends may be favourably altered. In the present study also it has been observed that even though, there is no significant increase in the percent grafting, the number of grafting sites significantly increases with thiolated and N-brominated collagen. An increase in the number of grafting sites was also observed in the case of vinylated collagen. It is known that unsaturated polymer can provide two separate sites for the growth of a branch or graft. The carbon atoms alpha to the double bond are particularly reactive and susceptible to attack by a free radical and by chain transfer to yield sites of grafting. In addition, the double bond can copolymerise with a vinyl monomer and thus serve as the site for the growth of branches. When collagen was vinylated by treatment with maleic anhydride and acrolein, the number of grafting sites was significantly increased in both cases but molecular weight of the grafted chains was much lower in the case of acrolein treated collagen. Similar results were also obtained in the case of cynoethylated collagen. Contrary to expectation, collagen treated with 10% oxidised dialdehyde starch showed only negligible grafting. Nayudamma *et al*[21,22] have recently reported that dialdehyde and polyaldehyde tanned collagen contain appreciable amount of free aldehyde groups. It is possible that the free aldehyde groups present in dialdehyde starch treated collagen may reduce the ceric ion and thus contribute to a decreased percentage of grafting. In the case of acrolein tanned collagen, free aldehyde groups were reported to be absent. In the oxidised starch treated collagen, the glucose units introduced may also act as poor chain transfer agents and thereby the percent grafting may be very low. It has already been observed by Kwei[23] that polyacrylic acid was grafted to DNA with greater ease compared to that of vinyl pyrolidine with dextran which also contained sugar units. This was attributed to the greatly increased chain transfer efficiency of sugar units in DNA in comparison with those in dextran.

Methylated and N-acetylated collagen gave the same percent of grafting as the control, but in the case of methylated collagen, there was a significant increase in the number of grafting sites. The reason for the increase in grafting sites in methylated collagen is not very clear. Probably in this case, the blocking of the carboxyl groups of collagen will prevent the chemisorption of ceric ions at the carboxyl groups of collagen and thus the availability of ceric ion for free radical liberation may be increased. It is known that with increasing catalyst concentration, the number of grafting sites increases while the molecular weight of the grafted chains decreases. In contrast to N-acetylated collagen, dinitrophenylated collagen did not graft any vinyl monomers. This may be explained by the fact that ceric salts rapidly oxidises some N-2-4-dinitrophenyl amines with the formation of 2,4-dinitraoniline and it is well established that aromatic nitro compounds such as dinitroaniline inhibits polymerisation. Haworth and Holker[24,25] also obtained similar results with dinitrophenylated nylon. In the case of deamination of collagen by nitrous acid, the amino groups are replaced by hydroxyl groups and as such it was expected that deamination may bring about an increase in the percent grafting. Contrary to expectation, however, there was a considerable decrease in the percent grafting even though the number of grafting sites was not significantly reduced as compared to untreated control collagen. Kuntzel[26] has reported that on deamination of collagen, aromatic nitro or nitroso compounds may also be formed through the participation of tyrosine residues and the retarding effect of these compounds in chain propagation may be responsible for the lower molecular weight of the grafted PMMA on deaminated collagen. The retarding effect of nitrobenzene in chain propagation has already been reported by Hamburger[27] Total acetylated collagen showed a decrease in percent grafting and also in the number of grafting sites. The data obtained on the grafting of vinyl monomers on different modified collagens do not provide any unequivocal proof on the nature of the functional group involved as grafting site. Ceric ion forms very effective redox systems in the presence of organic reducing agents such as alcohols, glycols, aldehydes, thiols, amines, esters and carboxylic acids.[28] Besides, amino, carboxyl and hydroxyl groups collagen also contains a few hexose residues. The presence of ester linkages[29,30] and aldehyde groups[31,32] in collagen has also been reported. Each molecule of collagen of approximately 300,000 molecular weight has been reported to contain about two to three aldehyde groups.[33] Since all these functional groups of collagen are able to form effective redox system with ceric ion, it is very difficult to determine which of these functional groups are involved in the grafting of the very small number of branches on the collagen molecule. The general trend of the results obtained, however, with modified collagen samples indicates that hydroxy groups and the peptide backbone may provide sites for initiation of grafting reaction. The slight reduction in the number of grafting sites observed in the case of N-acetylated collagen would suggest that amino groups may be involved

Table 2

EFFECT OF DIFFERENT TREATMENTS ON THE HYDROXYPROLINE CONTENT OF COLLAGEN

| Nature of treatment | Hydroxyproline content % |
|---|---|
| Nil-Tropocollagen | 13·60 |
| Tropocollagen treated with $HNO_3$ | 13.28 |
| Tropocollagen treated with CAN in $HNO_3$ | 13·17 |
| Tropocollagen grafted with PMMA* | 12·52 |
| Tropocollagen + PMMA (Physical Mixture) | 13·60 |

*Collagen content arrived from arginine value.

only to a very limited extent as grafting sites in collagen. The possibility of aldehydes or ester groups present in collagen acting as sites for grafting cannot also be ruled out from the present data. When PMMA grafted collagen was hydrolysed with 0·5N sulphuric acid, all the sugar residues present in collagen could be accounted for in the hydrolysate and no evidence was obtained for the presence of any sugar residue in the insoluble graft. This indicated that the hexose residues present in collagen are not involved as grafting site.

In our earlier studies[1,2] based on the results of hydroxyproline values of grafted collagen, it was suggested that this amino acid residue may probably be involved as grafting site. However, control experiments carried out in the present study (Table 2) indicate that the hydroxyproline values are also decreased in collagen by treating with the grafting medium without any vinyl monomer. Moudgal *et al*[34] have also reported that the hydroxyproline of elastoidin is destroyed by treatment with iodine. These results, together with the values obtained from the molecular weight determination of the grafted polymer chains would, therefore, indicate that hydroxyproline residues may not be involved to the extent suggested previously.

**Acknowledgment**

This research has been financed in part by a grant made by the US Department of Agriculture, Agricultural Research Service, under PL-480.

**REFERENCES**

1. Panduranga Rao, K., Thomas Joseph, K. & Nayudamma, Y., *Leath. Sci.*, **14**, 73 (1967).

2. Panduranga Rao, K., Thomas Joseph, K. & Nayudamma, Y., *Das Leder*, **19**, 77 (1968).

3. Imai, Y. & Iwakura, Y., *J., Appl. Polymer Sci.*, **11**, 1529 (1967).

4. Iwakura, Y. & Imai, Y., *J. Polymer Sci.* **C-23**, 461 (1968).

5. Bowes, J. H. & Kentan, R. H., *Biochem. J.*, **44**, 142 (1949).

6. Joseph, K. T. & Bose, S. M., *Bull. Cent. Leath. Res. Inst.*, **5**, 399 (1959).

7. Green, R. W., Ang, K. P. & Lam, L. C. *Biochem.J.*, **54**, 181 (1953).

8. Bose, S. M. & Thomas Joseph, K., *Arch. Biochem. Biophys.*, **74**, 46 (1958).

9. Sanger, F., *Biochem.J.*, **39**, 507 (1945).

10. Feairheller, S. H., Taylor, M., Gruber, H. A., Mellon, E. F. & Filachione, E. M., *J. Polymer Sci.*, **C-24**, 163 (1968).

11. Singer, S. J., Folhergill, J. E. & Shainoff, J. R., *J.Am.Chem.Soc.*, **82**, 565 (1960).

12. Bamford, C. H., Duncan, F. J., Reynolds, R. J. & Seddon, J. D., *J. Polymer Sci.*, **C-23**, 419 (1968).

13. Marten, J. & Speakman, J. B., *Chemistry and Industry*, 955 (1957).

14. Miller, L. A. & Whitfield, R. E., *Text. Res. J.*, **31**, 451 (1961).

15. Macpherson, H. T., *Biochem. J.*, **40**, 470 (1946).

16. Neuman, R. E. & Logan, M. A., *J. Biol. Chem.*, **184**, 299 (1950).

17. Fox, T. G., Kinsinger, J. B., Mason, H. F. & Schluele, E. M., *Polymer*, **3**, 71, (1962).

18. Michel Dubois, Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F., *Anal. Chem.*, **28**, 350 (1956).

19. Burlant, W. J. & Hoffman, A. S., *Block and Graft Polymers* Reinhold Publishing Corporation, New York, p. 24 (1960).

20. Ray Chaudhuri, D. K. & Hermans, J. J., *J. Polymer Sci.*, **48**, 159 (1960).

21. Nayudamma, Y., Joseph, K. T. & Bose, S. M., *J. Am. Leather Chemists Assoc.*, **56**, 548 (1961).

22. Nayudamma, Y., Hemalatha, R., Joseph, K. T., *J. Am. Leather Chemists Assoc.*, **64**, 444 (1969).

23. Kwei, K. P. Shen, *J. Poymer Sci.*, **A-1**, 2309 (1963).

24. Haworth, S. & Holker, J. R., *Nature*, **208**, 184 (1965).

25. Haworth, S., & Holker, J. R., *J.S.D.&C.*, **82**, 257 (1966).

26. Kuntzel, A., *Das Leder*, **8**, 34 (1957).

27. Hamburger, C. J., *J. Polymer Sci.*, **7**, 1023 (1969).

28. Mino, G. & Kaizerman, S., *J. Polymer Sci.*, **31**, 242 (1958).

29. Gallop, P. M., Seifter, S. & Meilmann, E., *Nature (Lond.)* **183**, 1659 (1959).

30. Hormann, H., *Leder*, **11**, 173 (1960).

31. Landucci, J. M., Pouradier, J. & Durante, M., in *Recent advances in Gelatin and Glue Research*, Ed. G. Stainsby, Pergmon Press, p. 62 (1958).

32. Gallop, P. M., *Biophys. J.*, **4**, 79 (1964).

33. Page, R. C. & Benidtt, E. P., *Laboratory Investigation*, **18**, 124 (1968).

34. Moudgal, N. R., Srinivasan, V. & Sarma, P. S., *J. Sci. Ind. Res.*, **15-C**, 67 (1956).